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Crucial aspects of high performance thin layer chromatography quantitative validation. The case of determination of rosmarinic acid in different matrices

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ABSTRACT

A new HPTLC method was envisaged to determine rosmarinic acid (RA) in different matrices with the aim of testing the influence of optimizing the main HPTLC operative parameters in view of a more stringent validation process. HPTLC LiChrospher silica gel 60 F254s, 20 cm × 10 cm, plates with toluene:ethyl formate:formic acid (6:4:1, v/v) as the mobile phase were used. Densitometric determinations were performed in reflectance mode at 330 nm. The method was validated giving rise to a dependable and high throughput procedure well suited to routine applications. RA was quantified in the range of 132–660 ng with RSD of repeatability and intermediate precision not exceeding 2.0% and accuracy within the acceptance limits. The method was tested on several commercial preparations containing RA in different amounts.

1. Introduction

Rosmarinic acid (RA) (Fig. 1), a natural phenolic compound found in many Lamiaceae herbs especially in rosemary and sage, is known for having a number of interesting biological activities, e.g. antiviral, antibacterial, anti-inflammatory, antioxidant [1]. Moreover RA has been recently reported to inhibit the hemorrhagic effect of snake venoms [2] and for its effects on Alzheimer's disease [3]. The main source of this compound is *Rosmarinus officinalis* L., the biological activities of its leaves extracts being well recognized [4].

Reports have been published on the TLC determination of RA in a variety of herbal extracts [5–10], none providing reliable quantitative results since the proposed methods were impaired by some methodological weakness.

Validation is a requirement, integrated in the development process, to demonstrate the reliability and the suitability of a quantitative method. In the last few years some papers dealing with TLC validation have been published especially in the field of pharmaceutical analysis [11–14]. In our opinion however, two critical aspects should be further emphasized in determinations concerned with herbal drug i.e. the claims of linearity and the calibration matrix effects.

In this paper these parameters were evaluated and the usefulness of a pre-validation step based on the accuracy profiles [15–19] was considered as a crucial requirement in developing a validated HPTLC method. The determination of rosmarinic acid in complex matrices is a good test-bed to assess the reliability of this appoach.

2. Experimental

2.1. Materials

RA standard was obtained from Carl Roth GmbH (Karlsruhe, Germany).

Commercial samples of *R. officinalis* L. hydro-alcoholic extracts, oleoresin and dried oleoresin were purchased locally.

The following diet supplements claiming to contain *Lamiaceae* dried extracts were analyzed: Rosmarino Capsule ERBAVITA®, Salvia Capsule ERBAVITA®.

All solvents and chemicals were of analytical grade. Water was purified by a Milli-Q-system (Millipore Corporation, Bedford, MA, USA).

2.2. Instrumentation

A Camag (Camag, Muttenz, Switzerland) HPTLC instrumental set-up consisting of sample applicator Linomat 5, TLC Scanner 3 and DigiStore 2 Documentation System was used for the analyses under the control of the software platform winCats 1.4.4 Planar Chromatography Manager (Camag).

HPTLC LiChrospher silica gel 60 F254s, 20 cm × 10 cm, (Merck, Darmstadt, Germany) were employed and developed in a Camag Automatic Developing Chamber ADC2 with the humidity control

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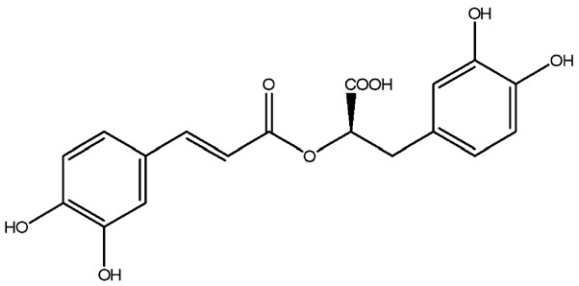

Fig. 1. Chemical structure of rosmarinic acid (RA).

option. Optimization of the separation conditions was carried out using a Camag HPTLC Vario System.

RA ESI mass spectra were obtained in negative ion mode by using the Camag MS-TLC interface linked to an HP 1100 MSD mass spectrometer with an API/electrospray interface (Agilent Technologies, Palo Alto, CA, USA).

2.3. Chromatographic procedure

The plates were pre-washed by dipping in CH_3OH over night, dried in N_2 stream under vacuum and stored in a clean environment. Standard and sample solutions were applied bandwise (bandlength 7 mm, 60 nL/s delivery speed, track distance 12.1 mm, distance from the edge 15 mm). Plates were developed with toluene:ethyl formate: formic acid (6:4:1, v/v) as the mobile phase (migration distance 80 mm), at room temperature, under humidity control (10 min, 38% relative humidity), 10 min plate preconditioning with the mobile phase and tank saturation.

The visual inspection and documentation of chromatograms were carried out at 254 nm and 366 nm. UV spectra were obtained in situ by the TLC Scanner 3. Densitometric determinations were performed in reflectance mode at 330 nm, D2 and W lamp slit dimension 5.00 mm × 0.45 mm, scanning speed 10 nm/s, data resolution 100 μm/step. Evaluation was by peak area measurement. Curve fitting was carried out with CurveExpert 1.4 software and for the statistical analysis Excel 2010 (Microsoft Office) was used.

2.4. Sample preparation

R. officinalis L. one-year old (A) (5 g) and new (B) (5 g) leaves were individually frozen in liquid N_2, very finely ground and then suspended in 50 mL of EtOH and sonicated in an ultrasonic bath for 30 min at 20 °C. The extracts were used as obtained.

Ten capsules of each of the diet supplements were finely ground and the amounts indicated were suspended in 2 mL of EtOH and sonicated in an ultrasonic bath for 15 min at 20 °C: Rosmarino Capsule ERBAVITA® (304.8 mg), Salvia Capsule ERBAVITA® (300.76 mg).

The oleoresin (E) (302.19 mg) and dried oleoresin (F) (301.25 mg) were suspended in 2 mL of EtOH–acetone 1:1 (v/v).

The resulting suspensions were centrifuged for 5 min at 10,000 rpm and the supernatant used as obtained.

The hydro-alcoholic extracts (C, D) were diluted with EtOH 1:2 (v/v).

All samples were stored under N_2 in dark vials at 4 °C and used within a week.

For the assay 3 μL of these solutions were applied.

2.5. Calibration standards

Rosmarinic acid standard solution (1.32 mg/mL) was prepared by dissolving 13.20 mg in 10 mL of EtOH.

For the validation step four calibration EtOH solutions (44.0, 88.0, 132.0 and 220.0 ng/μL) were obtained from the standard solution using the Serial Dilution option of the Perkin-Elmer HPLC Autosampler Series 200 (Perkin-Elmer, Walthman, MA, USA). For the calibration curves 3 μL of these solutions were applied.

Three validation standard solutions were prepared individually spiking 10 mL of a diluted 1:16 EtOH extract A with 1.056, 1.716 and 2.376 mg of RA.

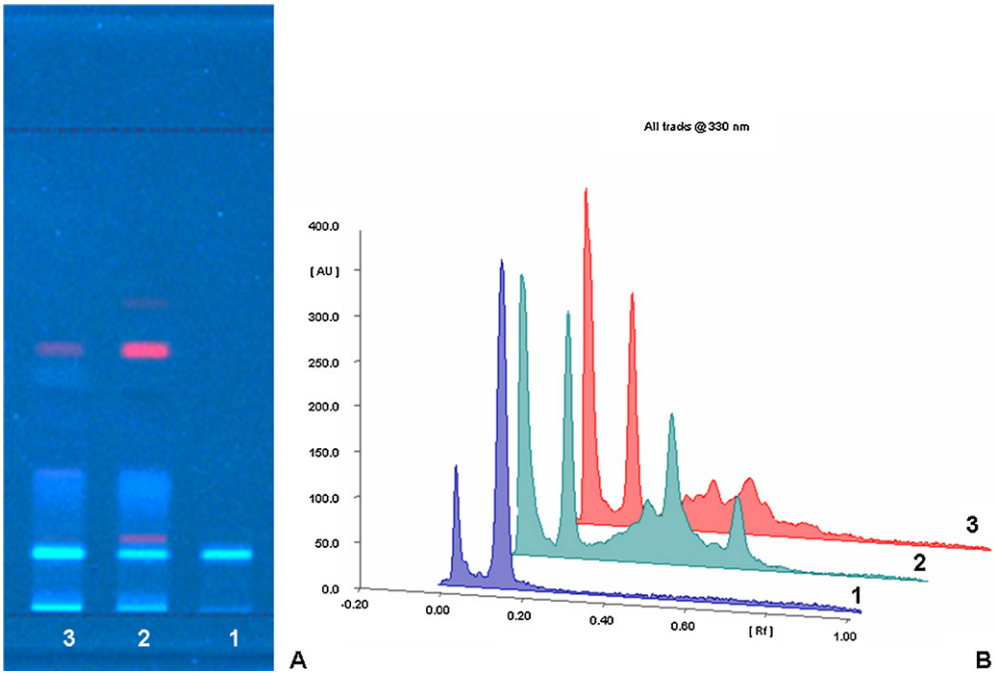

Fig. 2. (A) HPTLC videodensitograms at 365 nm and (B) densitogram at 330 nm of RA commercial standard (1) and *R. officinalis* ethanolic extracts A (2) and B (3) from fresh leaves.

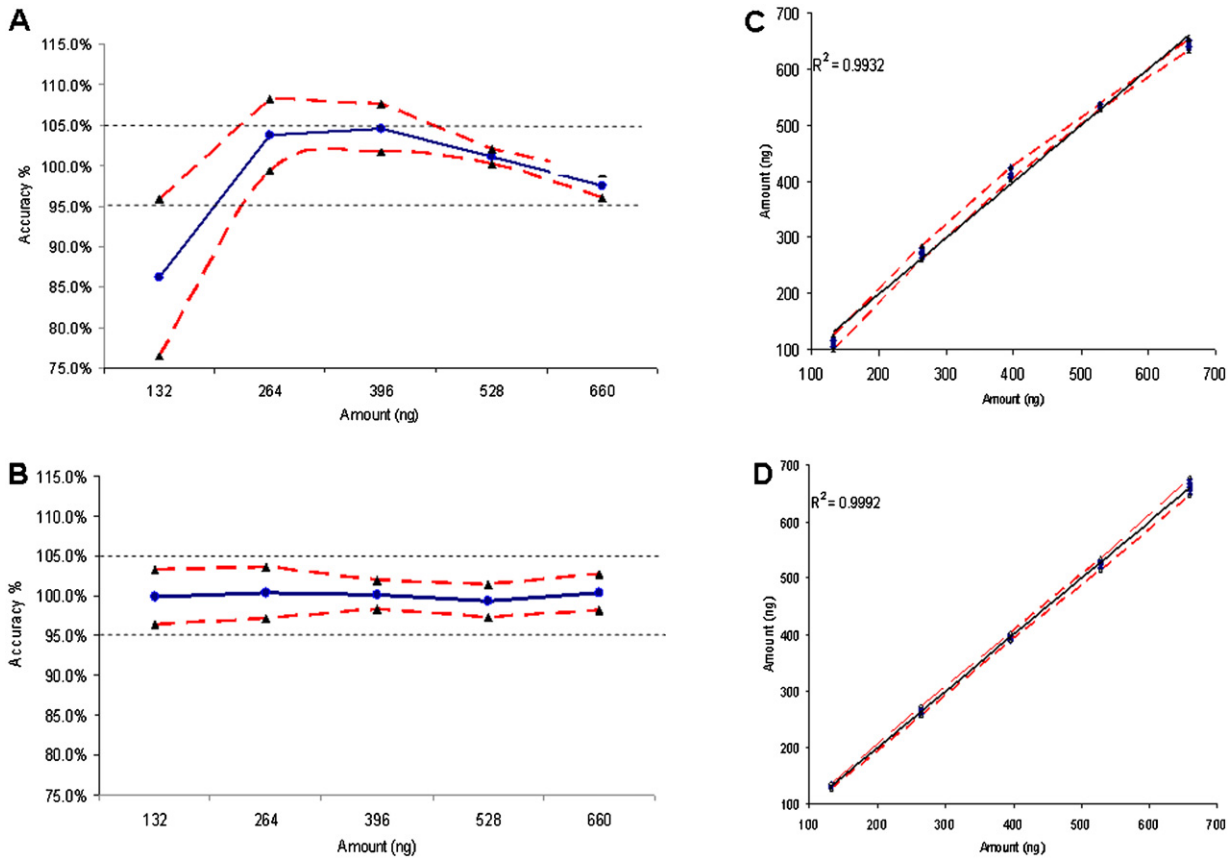

Fig. 3. Accuracy profiles of RA obtained with (A) linear regression model, (B) weighed ($1/x$) quadratic model. ♦ Confidence intervals, ● relative trueness. Linearity of results with linear regression model (C) and weighed ($1/x$) quadratic model (D). –– confidence intervals.

2 μL of these solutions were applied onto the plates obtaining a RA total amount of 282.83, 414.83 and 546.83 ng.

All standards were stored under N_2 in dark vials at 4 °C and used within a week.

2.6. Standard addition method

Five calibration standard solutions were obtained spiking a 10 mL of an EtOH diluted extract A (RA = 26.60 μg/mL) with 0.40, 1.06, 1.72, 2.38 and 3.04 mg of RA. For the calibration curves 2 μL of these solutions were applied.

3. Results and discussion

3.1. Method optimization

Several eluent systems and chromatographic conditions, comprising those reported in the literature [20,21], were tried in order to separate RA from the co-occuring compounds and to obtain a fingerprint of *R. officinalis* L. ethanolic extract. After thoroughly testing the most satisfactory resolution was obtained with toluene:ethyl formate:formic acid (6:4:1, v/v) as the mobile phase, in saturated mode (Fig. 2).

The specificity of the method was assessed before starting the validation step. A peak purity test of RA in each sample was performed comparing the UV overlaid spectra measured within the RA peak in both the peak flanks and at peak maximum. No interference was observed regarding the densitograms of the samples, confirming the selectivity of the method. Moreover, the identity of RA was confirmed by the mass spectrum obtained in situ with HPTLC–MS interface. In our operative conditions, RA ESI-MS in negative ion mode gives rise to a spectrum characterized by the spontaneous fragmentation pattern $[M-H]^-$ m/z 359 (18%); m/z 197 (55%); m/z 179 (18%); m/z 161 (100%); m/z 135 (15%), in full agreement with the CID fragmentation pattern reported in the literature [22].

The analyte was tested for stability during development performing a two-dimensional separation [23]. Moreover, it was demonstrated that the samples were stable in solution for more than a week and on the plate for not less than 12 h before and after development.

3.2. Validation

According to the definition of validation, the acceptance criteria should be taken into account and tailored to the intended use of the analytical method. None acceptance criteria are provided for the determination of active compounds in natural products. Nowadays however, when a method concerned with assaying an active ingredient in herbal matrices and diet supplements is designed, repeatability, intermediate precision and accuracy set at ±5% or better (far lower to the 15% FDA guidelines for bioanalysis and complex matrices [24]) is in our opinion a target considering the instrumental performances currently available.

In a pre-validation step, the selection of the right calibration model was achieved by means of the accuracy profiles based on the total error and confidence interval. The use of this procedure is a good opportunity in HPTLC-densitometry where the measurements, carried out in reflectance mode following the Kubelka–Munk equation instead of the Lambert–Beer law, give a seldom verifiable linearity and in a very narrow range.

Table 1
Validation results. Calibration standard response functions ($n = 12$).

	Day 1	Day 2	Day 3
Intercept	−522.8	−726.4	−655.5
Slope	15.58	17.98	15.73
Quadratic term ($\times 10^{-3}$)	−7.21	−8.1	−6.3
R^2	0.99970	0.99985	0.99988

Trueness ($n = 9$)				Precision ($n = 9$)	
Standard amount (ng)	Absolute bias (ng)	Relative bias (%)	Recovery (%)	Repeatability (RSD %)	Intermediate precision (RSD %)
132	−0.03	−0.02	99.98	2.01	1.66
264	0.19	0.07	100.07	0.88	1.05
396	−0.22	−0.06	99.94	1.09	1.12
660	0.14	0.02	100.02	1.27	1.05

Linearity of the results	
Range (ng)	132–660
Intercept	−0.0323
Slope	1.0001
R^2	0.9995

Adverse effects of overloading are to be carefully avoided in planar chromatography. Accordingly the calibration range should be selected as low as possible, also starting near the LOQ. In this way, the lowest level of the calibration curve coincides with the limit of quantitation (LOQ).

Five calibration levels were obtained in triplicate and repeated on three different days over a range of 132–660 ng of the analyte. Linear model, weighed linear ($1/x$) model, quadratic model and weighed ($1/x$) quadratic model were the regression functions taken into consideration and used to back-calculate the mean bias, the repeatability and the intermediate precision for each level. Although the regression data for the linear model ($n = 15$) seemed to be adequate ($R^2 = 0.9974$; $y = 60.43 + 10.8x$; standard error = 150), its accuracy profile clearly showed the linear model not suitable, producing nonlinear results (Fig. 3). The accuracy profiles obtained allowed to select the weighed quadratic ($1/x$) model as the best choice giving linear results, in agreement with the ICH linearity definition: "The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample" (Fig. 3).

One of the major advantages of TLC is the minimal sample preparation normally required. However the existence of a possible matrix effect should be constantly taken into account. When no blank matrix is available, the method of standard addition is to be used. The standard addition curve, assembled within the calibration range and calculated with the weighed ($1/x$) quadratic regression function, coincided with the calibration curve obtained for the pure analyte in absence of matrix, assessing the validity of the calibration model ($y = -0.00543x^2 + 15.26x - 500.66$; $R^2 = 0.9997$ and $y = -0.00858x^2 + 17.12x + 506.02$; $R^2 = 0.9994$, respectively).

Four points calibration curves resulted adequate for routine analysis; as a consequence, the amount levels 132, 264, 396 and 660 ng were selected for the validation curves. Accordingly, four calibration points without the matrix were obtained in triplicate on three different days. As stated in the pre-validation experiments a weighed ($1/x$) quadratic model was used. The validation results are shown in Table 1. The accuracy profile, largely inside the acceptance limits (±5%) proved that the method fulfilled the requirements (Fig. 4).

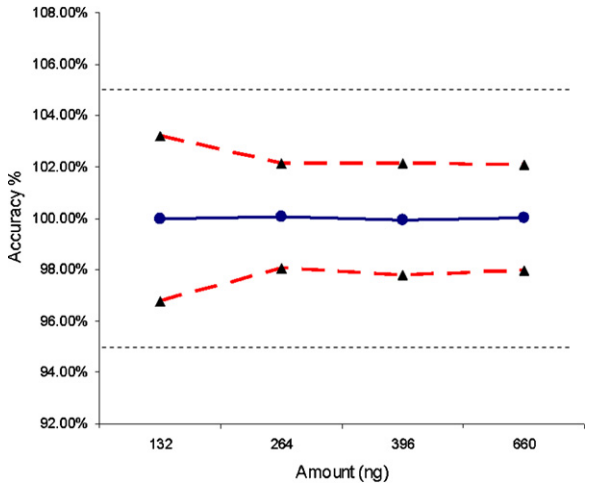

Fig. 4. Accuracy profile of RA in the validated method.

Since no alternative validated method was available, for accuracy purpose three validation standards were obtained spiking a diluted EtOH extract A. The amounts of RA, suitable to obtain values inside the calibration range, were added.

The accuracy values were obtained in triplicate on three different days at three amount levels 282.83, 414.83 and 546.83 ng. The accuracy results are shown in Table 2.

When testing the robustness of a HPTLC method based on silica gel plates, relative humidity and saturation conditions are crucial parameters affecting the Rf, the relative retention and sometime even the elution order. The constant use of an automatic developing chamber with the humidity control option, where those parameters are under control, overcomes these difficulties. Therefore,

Table 2
Accuracy results.

Matrix amount (ng)	Added amount (ng)	Total amount (ng)	Mean result (ng)	Absolute bias (ng)	Relative bias (%)	Recovery (%)	RSD (%)
71.63	211.20	282.83	289.12	6.29	2.22	102.22	2.94
	343.20	414.83	427.15	12.31	2.97	102.97	1.67
	475.20	546.83	562.94	16.11	2.95	102.95	2.86

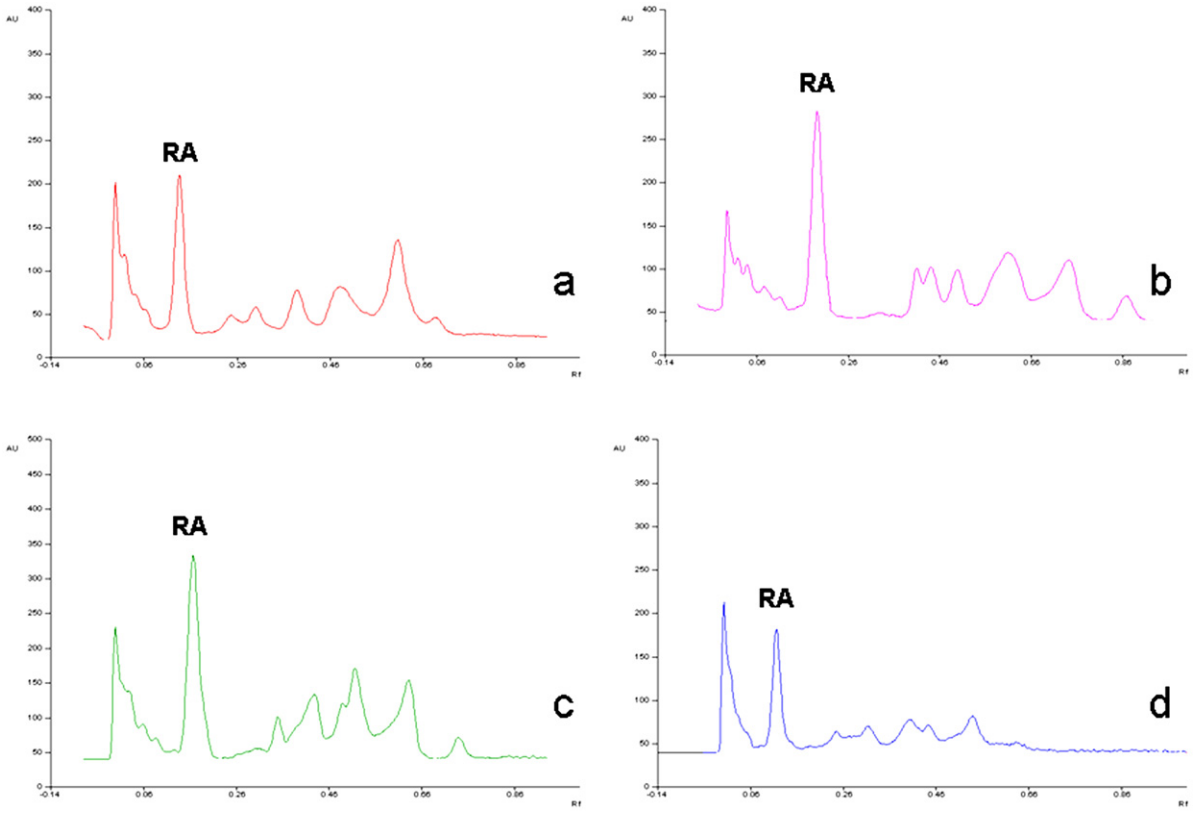

Fig. 5. HPTLC densitogram at 330 nm of *R. officinalis* ethanolic extracts B from fresh leaves using (a) Silica gel 60 F254s, run 80 mm, chamber saturation, plate preconditioning; (b) LiChrospher Si 60 F254s, run 80 mm, chamber saturation; (c) LiChrospher Si 60 F254s, run 75 mm, chamber saturation; (d) LiChrospher Si 60 F254s, run 80 mm, chamber saturation, plate preconditioning.

Table 3
Robustness test on the 414.83 ng validation standard solution.

Conditions		Mean peak area ($n = 3$)	RSD (%)
Stationary phase	LiChrospher Si 60 F254s	5128.94	0.80
	Silica Gel 60 F254s	2854.30	1.06
Developing distance	75 mm run	6648.07	0.48
	80 mm run	6108.70	1.40
Saturation conditions	Saturated chamber	5128.94	0.80
	Unsaturated chamber	5247.90	1.14

having verified that the results were substantially unaffected by minor changes in eluent composition (Fig. 5), the robustness test was built up taking into account the following items: different silica gel layers, plate saturation conditions and developing distances (Table 3).

Table 4
Assay for RA in real samples.

Sample	Found ($n = 6$) (ng)	RSD (%)
Ethanolic extract A	431.2[a]	1.83
Ethanolic extract B	488.0[a]	1.84
Hydroalcholic extract C	317.6[a]	1.31
Hydroalcholic extract D	326.4[a]	0.68
Rosmarino capsules	756.0[b]	4.01
Salvia capsules	370.4[b]	4.72
Oleoresin E	860.0[b]	1.66
Oleoresin F	450.0[b]	2.92

[a] Referred to 1 mL of sample.
[b] Referred to 100 mg of sample.

4. Conclusions

This study was undertaken in order to test the influence of optimizing the main HPTLC operative parameters in view of a more stringent validation process. Together with the fundamental items of the HPTLC instrumental set-up, the Automatic Developing Chamber (ADC) was confirmed as mandatory to control the relative humidity and the saturation conditions, both crucial to assure the required run to run reproducibility. Consequently, repeatability, intermediate precision and accuracy largely inside the acceptance limits (±5%) were attained. The method was applied to determine RA content in a variety of different samples. The results are reported in Table 4.

References

[1] M. Petersen, M.S. Simmonds, Phytochemistry 62 (2003) 121.
[2] H.T. Aung, T. Nikai, M. Niwa, Y. Takaya, J. Nat. Med. 64 (2011) 482.
[3] T. Hamaguchi, K. Ono, A. Murase, M. Yamada, Am. J. Pathol. 175 (2009) 2557.
[4] ESCOP Monographs, The Scientific Foundation for Herbal Medicinal Products, 2nd ed., Rosmarini Folium Thieme, 2009.
[5] I. Fecka, S. Turek, Food Chem. 108 (2008) 1039.
[6] I. Fecka, S. Turek, J. Agric. Food Chem. 55 (2007) 10908.
[7] I. Bros, M.-L. Soran, R.D. Briciu, S.C. Cobzac, J. Planar Chromatogr. 22 (2009) 25.
[8] I. Fecka, D. Raj, M. Krauze-Baranowska, Chromatographia 66 (2007) 87.
[9] J. Nowakowska, J. Planar Chromatogr. 17 (2004) 149.
[10] G. Janicsak, I. Mathe, Acta Pharm. Hung. 68 (1998) 259.
[11] B. Renger, Z. Vegh, K. Ferenczi-Fodor, J. Chromatogr. A 1218 (2011) 2712.
[12] K. Ferenczi-Fodor, B. Renger, Z. Vegh, J. Planar Chromatogr. – Mod. TLC 23 (2010) 173.
[13] K. Ferenczi-Fodor, Z. Vegh, A. Nagy-Turak, B. Renger, M. Zeller, J. AOAC Int. 84 (2001) 1265.
[14] K. Koll, E. Reich, A. Blatter, M. Veit, J. AOAC Int. 86 (2003) 909.
[15] E. Rozet, V. Wascotte, N. Lecouturier, V. Preat, W. Dewe, B. Boulanger, P. Hubert, Anal. Chim. Acta 591 (2007) 239.

[16] P. Hubert, J.J. Nguyen-Huu, B. Boulanger, E. Chapuzet, N. Cohen, P.A. Compagnon, W. Dewe, M. Feinberg, M. Laurentie, N. Mercier, G. Muzard, L. Valat, E. Rozet, J. Pharm. Biomed. Anal. 45 (2007) 82.
[17] P. Hubert, J.J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.A. Compagnon, W. Dewe, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G. Muzard, C. Nivet, L. Valat, E. Rozet, J. Pharm. Biomed. Anal. 45 (2007) 70.
[18] M. Feinberg, J. Chromatogr. A 1158 (2007) 174.
[19] P. Hubert, J.J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.A. Compagnon, W. Dewe, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G. Muzard, C. Nivet, L. Valat, J. Pharm. Biomed. Anal. 36 (2004) 579.
[20] H. Wagner, S. Bladt, Plant Drug Analysis a Thin Layer Chromatography Atlas, 2nd ed., Springer-Verlag, Berlin, 1996.
[21] European Pharmacopoeia, European Council, 5th ed., Strasbourg, 2005.
[22] M.B. Hossain, D.K. Rai, N.P. Brunton, A.B. Martin-Diana, C. Barry-Ryan, J. Agric. Food Chem. 58 (2010) 10576.
[23] E. Reich, A. Schibli, High-Performance Thin-Layer Chromatography for the Analysis of Medicinal Plants, Thieme Medical Publishers Inc., New York, NY, 2006.
[24] Guidance for Industry: Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER), Rockville, 2001.